Serum 25-Hydroxyvitamin D and Breast Cancer in the Military: A Case-control Study Utilizing Pre-diagnostic Serum

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Naval Health Research Center

Report No. 12-21

This research was conducted in compliance with all applicable federal regulations governing the protection of human subjects in research.

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Cancer Causes Control (2013) 24:495–504
DOI 10.1007/s10552-012-0140-6

ORIGINAL PAPER

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Received: 12 June 2012 / Accepted: 21 December 2012 / Published online: 8 January 2013

Abstract
Purpose The objective of this study was to ascertain whether a relationship exists between pre-diagnostic serum levels of 25-hydroxyvitamin D (25(OH)D) and risk of breast cancer in young women.
Methods About 600 incident cases of breast cancer were matched to 600 controls as part of a nested case–control study that utilized pre-diagnostic sera. Logistic regression was used to assess the relationship between serum 25(OH)D concentration and breast cancer risk, controlling for race and age.
Results According to the conditional logistic regression for all subjects, odds ratios for breast cancer by quintile of serum 25(OH)D from lowest to highest were 1.2, 1.0, 0.9, 1.1, and 1.0 (reference) (p trend = 0.72). After multivariate regression for subjects whose blood had been collected within 90 days preceding diagnosis, odds ratios for breast cancer by quintile of serum 25(OH)D from lowest to highest were 3.3, 1.9, 1.7, 2.6, and 1.0 (reference) (p trend = 0.09).
Conclusions An inverse association between serum 25(OH)D concentration and risk of breast cancer was not present in the principal analysis, although an inverse association was present in a small subgroup analysis of subjects whose blood had been collected within 90 days preceding diagnosis. Further prospective studies of 25(OH)D and breast cancer risk are needed.

Keywords Vitamin D · Breast neoplasms · Case–control studies · 25-hyrdoxyvitamin D · Epidemiology

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Introduction

Breast cancer is the most commonly occurring neoplasm among women in the United States with an estimated 226,870 new cases and 39,510 deaths occurring in 2012 [1]. Previous research has shown high levels of sunlight or ultraviolet B radiation (UVB) [2–7] in the environment are associated with lower incidence and mortality from breast cancer. Exposure to UVB results in the photosynthesis of vitamin D_3 in the skin [2, 4, 8–11] which is enzymatically converted in the liver to 25-hydroxyvitamin D [25(OH)D], the principal circulating vitamin D metabolite, in a dose-dependent manner [12]. These findings led to the hypothesis that higher levels of 25(OH)D may reduce a woman's risk of developing breast cancer [2, 8, 13].

In addition to the protective effect of increased UVB radiation exposure regarding breast cancer, a beneficial effect of increased intake of supplemental vitamin D has also been demonstrated [14–18]. Several cancers including

those of the breast [19–27], colon [28], and ovary [29] have been linked to the main marker of vitamin D deficiency: low levels of serum 25(OH)D [30].

Several epidemiological studies of the effect of 25(OH)D levels on breast cancer risk have been conducted to test the vitamin D–breast cancer theory. So far, the evidence in favor of vitamin D has been supportive. Eight epidemiological studies found a significant inverse associations between higher levels of serum 25(OH)D and estimated risk of breast cancer [19–27], while five failed to detect a significant association [22, 31–34].

Furthermore, several recent meta-analyses found that higher serum concentrations of 25(OH)D were significantly associated with lower risk of breast cancer for all studies combined and included in a pooled analysis of published studies [35–38].

The main objective of this study was to test whether a relationship exists between pre-diagnostic serum levels of 25-hydroxyvitamin D (25(OH)D) and subsequent risk of breast cancer. This study will also quantify the dose–response relationship between serum 25(OH)D and subsequent risk of breast cancer using previously collected stored sera. This will allow for the determination of the optimal circulating 25(OH)D levels needed to reduce breast cancer risk.

The majority of studies that have found an inverse association between serum 25(OH)D concentration and risk of breast cancer have been case–control studies in which 25(OH)D was measured during or shortly before diagnosis [19, 20, 22, 23, 25–27], while most studies that have failed to detect a significant relationship are nested case–control studies where serum 25(OH)D levels are often measured years before diagnosis [22, 31–34]. This indicates that the ability to detect a statistically significant protective effect of 25(OH)D on risk of breast cancer may be modified by the time lag between 25(OH)D measurement and case diagnosis. In order to investigate this possible interaction, an analysis stratified by length of time between 25(OH)D measurement and case diagnosis, using several cut points for the lag time, was performed.

Methods

Setting

This study employed a case–control design and utilized pre-diagnostic serum collected from 600 incident cases of female breast cancer and 600 controls. Serum was collected from all active-duty members of the US military as part of a comprehensive serum screening program conducted during 2002–2008. The samples were collected for military health surveillance and epidemiologic research, were frozen into 0.5 ml aliquots, and stored in the Department of Defense Serum Repository [39]. The data were stored in the Defense Medical Surveillance System (DMSS), the central repository of medical surveillance data for the US Department of Defense. This secure repository contains current and historical data on hospitalizations and outpatient medical encounters during the military career and demographic information. It has been used previously for epidemiological research [39].

Participants

Cases were ascertained from a comprehensive database assembled from all Department of Defense (DoD) medical treatment facilities and Tricare civilian hospitals worldwide. A case was identified as an active-duty service military member who was diagnosed with breast cancer (ICD 9-CM 174.0–174.9). Both the case and control were on active-duty military service during 1994–2009. The case definition required (a) being hospitalized with a discharge diagnosis of breast cancer certified by a physician or (b) 3 or more outpatient medical care visits with a primary diagnosis of breast cancer. Controls were individually matched to cases according to the date the blood sample was drawn (±2 days), age (±1 year), length of service (±30 days), and whether the control was on active duty on the date the case was diagnosed.

The most recent serum sample preceding the date of diagnosis of breast cancer was obtained for each case and its matched control. If more than one potential control met the criteria, the one whose date of serum collection was closest to that of the case was selected.

Laboratory procedures

Blood samples were collected in plain tubes, allowed to clot, and serum was separated from cells using routine centrifugation. Serum was divided into 0.5 ml aliquots in polypropylene cryogenic vials and frozen at −70°C. It was stored in Revco freezers equipped with temperature alarms. Sera were analyzed by a major laboratory (Ames IA: Heartland Assays), using the Diasorin LIAISON radioimmunoassay. This method is an FDA-approved direct, competitive chemiluminescence immunoassay (CLIA) using the DiaSorin LIAISON 25-OH vitamin D total assay [40, 41]. This is a gold standard method for the measurement of the serum 25(OH)D concentration [41]. It uses an antibody to 25-hydroxyvitamin D to coat magnetic particles (solid phase), and a vitamin D analog, 22-carboxy-23,24,25,26,27-pentanorvitamin D_3, linked to an isoluminol derivative, making it capable of chemiluminescence. 25(OH)D dissociates from its binding protein in the serum during an incubation period. It then competes with the isoluminol-labeled analog for binding sites on the antibody. After the incubation, the unbound material is washed away.

Further reagents are added and a chemiluminescent reaction begins. The intensity of the light produced is measured by a photomultiplier sensor and expressed as relative light units (RLU). Since smaller concentrations of 25(OH)D displace fewer isoluminol-labeled molecules of the vitamin D analog from the antibody, the intensity of the light is inversely proportional to the concentration of 25-hydroxyvitamin D. The inter-and intra-assay coefficients of variation of this assay are 12.4 and 5.4 %, respectively, the lower limit of detection is 2.5 ng/ml [41]. The laboratory validated its measurements by testing standard aliquots of 25(OH)D provided by the Vitamin D External Quality Assessment Scheme (DQAS), a nonprofit 25(OH)D calibration program [42]. The laboratory had no knowledge of case or control status of the samples.

Statistical analysis

Analyses were performed in order to assess differences between cases and controls with respect to mean 25(OH)D concentration and distribution of race, rank, and age, without adjustment for other covariates. Conditional logistic regression was employed to control for confounding in the multivariate analysis. Quintiles of serum 25(OH)D concentration were defined based on the distribution of 25(OH)D concentrations in the control population. Odds ratios were determined using the highest quantile of serum 25(OH)D concentration in the control population as the reference category. The criterion for statistical significance of the association of 25(OH)D with risk of breast cancer was $p \leq 0.05$, two-tailed. Logistic regression analysis was performed using the PROC LOGISTIC procedure in SAS 9.3. The p for trend values was calculated using the Wald chi-square test. Median 25(OH)D values of the quintiles were calculated from continuous 25(OH)D levels in each quintile.

In order to investigate the possible interaction between ability to detect a protective effect of 25(OH)D on risk of breast cancer and time lag between 25(OH)D measurement and case diagnosis, a sensitivity analysis was performed in which the conditional logistic regression analyses were repeated after stratifying on length of time between 25(OH)D measurement and case diagnosis using the following cut points: $\geq$90 days, $\geq$180 days, $\geq$1 year and $\leq$90 days, $\leq$180 days, and $\leq$1 year.

As a further sensitivity analysis, the dose–response relationship between serum 25(OH)D and estimated breast cancer risk was estimated after limiting the study population to white pairs and also to white pairs in which case diagnosis was preceded by blood draw <90 days. A dose–response curve using a least-squares line was plotted showing the odds ratios for each quintile of the unmatched, unadjusted data, using individuals in the highest quintile of 25(OH)D concentration as the reference group. p values for trend were calculated using the Mantel–Haenszel chi-square test [43]. The same analyses were repeated for nonwhite pairs. This study was conducted in accordance with the ethical standards of the relevant Department of Defense Institutional Review Board (IRB) and the Declaration of Helsinki 1975, as revised in 1983, and IRB approval was obtained. All analyses were performed using SAS (version 9.2) (Cary NC: SAS Institute).

Results

There were 600 matched pairs consisting of a case of breast cancer and her healthy control. In univariate analyses, cases did not differ significantly from controls with respect to mean 25(OH)D levels, distribution of race, rank, or age (Table 1). Median time between serum 25(OH)D measurement and case diagnosis was 299 days (10th percentile = 833.5 days; 90th percentile = 39 days).

In a multivariate conditional logistic regression model of 600 matched pairs, the relationship between serum 25(OH)D and odds of breast cancer was not statistically significant (Table 2), after adjusting for race. Odds ratios by quintile of 25(OH)D level were 1.2, 0.98, 0.95, 1.12, and 1.0 (reference) (p for trend = 0.72). In this analysis, race was not significantly associated with odds of breast cancer.

In the sensitivity analysis of pairs in which blood draw preceded case diagnosis by up to 90 days, odds ratios for breast cancer by quintile of serum 25(OH)D from lowest to highest were 3.3, 1.9, 1.7, 2.6, and 1.0 (reference) (p trend = 0.09). When the analysis was restricted to pairs

Table 1 Characteristics of cases and controls

Variable	Cases (n = 600)	Controls (n = 600)	p value
25(OH)D ng/ml			
Mean (SD)	24.8 (12.2)	25.9 (12.3)	0.13*
Race (%)			
White	314 (52.4)	350 (58.3)	
Black	216 (36)	186 (31)	
Other	70 (11.6)	64 (10.7)	0.11†
Rank (%)			
Enlisted	370 (61.7)	368 (61.3)	
Officer	230 (38.3)	232 (38.7)	0.91†
Age in years (%)			
20–35	156 (26)	152 (25.3)	
36–40	160 (26.6)	164 (27.3)	
41–45	159 (26.6)	156 (26)	
>45	125 (20.8)	128 (21.4)	0.98†

* t test, two-tailed

† X^2 test

in which blood draw preceded case diagnosis by greater than 90 days, there was no statistically significant relationship (Table 3).

Table 2 Conditional logistic regression analysis of serum 25(OH)D and risk of breast cancer, 600 matched pairs, controlling for race

Covariate	No. of cases/ controls	Regression coefficient	Odds ratio	95 % CI	*p*
Serum 25(OH)D ng/ml					
≤14.9	147/120	0.1737	1.19	0.8, 1.8	0.42
15–21.7	113/119	−0.0207	0.98	0.7, 1.4	0.92
21.8–27.3	108/121	−0.0567	0.95	0.6, 1.4	0.77
27.4–35.1	123/120	0.1198	1.12	0.8, 1.6	0.52
≥35.2 (reference group)	109/120	–	1.0	–	–
p for trend			0.72		

In an unadjusted, unmatched analysis of white subjects (*p* trend = 0.39) (Fig. 1) and nonwhite subjects (*p* trend = 0.77) (Appendix Fig. 3), there was no statistically significant relationship between serum 25(OH)D concentration and risk of breast cancer. In an unmatched, unadjusted subgroup analysis of white subjects in which case diagnosis of the case was preceded by blood draw by ≤90 days, there was a trend toward an inverse, linear relationship that did not reach statistical significance (Fig. 2). When similar analysis was done of nonwhite individuals (*p* trend = 0.02) (Appendix Fig. 4) and all subjects combined (*p* trend = 0.04), there were a significant, inverse, linear dose–response relationships (Appendix Fig. 5).

Discussion

In a population of mostly premenopausal women, there was no statistically significant relationship between serum

Table 3 Conditional logistic regression analysis of serum 25(OH)D level and risk of breast cancer according to the length of time between serum 25(OH)D measurement and case diagnosis, controlling for race, matching retained

Mean serum 25(OH)D ng/ml	≤90 days		≤180 days		≤365 days	
	n = 124 pairs		*n* = 216 pairs		*n* = 365 pairs	
	OR	95 % CI	OR	95 % CI	OR	95 % CI
≤14.9	3.3	1.6, 7.1	1.5	0.8, 3.1	1.3	0.7, 2.2
15–21.7	1.9	0.9, 4.1	0.8	0.4, 1.6	0.8	0.5, 1.4
21.8–27.3	1.7	0.8, 3.7	1.4	0.7, 2.7	1.0	0.6, 1.6
27.4–35.1	2.6	1.2, 5.4	1.4	0.8, 2.7	1.1	0.7, 1.8
≥35.2 (reference group)	1	–	1.0	–	1.0	–
p trend	0.09		0.25		0.54	
Race						
Black	1.1	0.8, 1.5	1.1	0.7, 1.5	1.2	0.8, 1.9
Other	1.2	0.8, 1.9	1.2	0.7, 2.1	1.1	0.6, 2.1
White (reference group)	1	–	1	–	1	–
	≥90 days		≥180 days		≥365 days	
	n = 479 pairs		*n* = 384 pairs		*n* = 235 pairs	
	OR	95 % CI	OR	95 % CI	OR	95 % CI
≤14.9	1.0	0.6, 1.6	1.1	0.6, 1.8	1.1	0.6, 2.1
15–21.7	1.3	0.6, 1.4	1.1	0.7, 1.7	1.2	0.7, 2.3
21.8–27.3	1.0	0.6, 1.3	0.8	0.5, 1.3	1.0	0.5, 1.7
27.4–35.1	0.9	0.6, 1.4	1.0	0.6, 1.5	1.1	0.6, 2.0
≥35.2 (reference group)	1.0	–	1.0	–	1.0	–
p trend	0.94		0.69		0.91	
Race						
Black	1.2	0.9, 2.0	1.6	0.9, 2.6	1.2	0.8, 1.8
Other	1.2	0.5, 2.9	1.3	0.7, 2.5	1.3	0.8, 2.2
White (reference group)	1	–	1	–	1	–

25(OH)D and breast cancer risk when all matched pairs were analyzed. In a subgroup analysis of pairs in which serum 25(OH)D measurement preceded case diagnosis by ≤90 days, women in the lowest quintile of 25(OH)D had 3.3 (95 % CI 1.6–7.1) times the estimated risk of breast cancer as those in the highest quintile. On the other hand, it is important to note that overall, there was not a statistically significant trend across quintile-specific ORs. In addition, the small sample size in the subgroup analysis ($n = 124$) resulted in wide confidence intervals.

In other models in which serum 25(OH)D measurement preceded case diagnosis by greater than 90 days, the relationship was not statistically significant (Table 3), possibly because the longer the time interval between 25(OH)D measurement and diagnosis, the less representative the measurement is likely to be of 25(OH)D levels during the most relevant window of time for cancer prevention, which may possibly be the last 3 months preceding diagnosis of breast cancer.

One possible explanation for this finding is that the serum 25(OH)D during the 3-month interval preceding diagnosis is physiologically critical to the growth of the tumor [44]. If so, earlier levels of 25(OH)D, collected at more remote points in time, would not be as associated with risk of breast cancer as those collected at the immediate stage of tumor growth preceding diagnosis [44]. This is likely to be the point at which the tumor may be most actively recruiting blood vessels [44]. Blood samples drawn earlier would have more statistical noise than those taken at the critical point that 25(OH)D most actively inhibits neoangiogenesis [45]. In a previous study performed by Rejnmark et al., serum 25(OH)D levels were measured approximately 1 month before case diagnosis. In that study, a statistically significant 48 % lower risk was observed in women in the highest tertile of 25(OH)D concentration compared to women in the lowest.

An alternative explanation for the inverse associations observed in case–control studies is reverse causality. In other words, the tumor itself could be responsible for lower serum 25(OH)D levels, possibly by consuming the metabolite, although there is no evidence known to the authors that such a phenomenon exists. Another explanation is that behavioral changes resulting from underlying breast cancer may cause diminished sunlight exposure due to a woman feeling unwell. However, nearly 80 % of incident cases of breast cancer are discovered as a result of self-examination when a lump is found in the breast or armpit [46]. Therefore, most incident cases lack the severe symptoms characteristic of a more advanced stage of the disease that might be likely to cause a drastic change in lifestyle habits.

Perhaps, unexpectedly, higher premenopausal BMI has been associated with decreased risk of breast cancer in premenopausal women [47]. High BMI was associated with more frequent anovulatory cycles, which are more common in overweight or obese women [48, 49]. Not controlling for BMI in the present study could have made low 25(OH)D concentrations seem less strongly associated than they were. If this study had controlled for BMI, the association could possibly have been more strongly inverse than that reported here. On the other hand, the military has strict physical fitness standards that members must meet in order to remain on active duty. The allowable upper limit of BMI in males is 27 kg/m^2 and in females is 26 kg/m^2 [50].

The mechanisms by which vitamin D may prevent breast cancer are still not completely understood. High serum levels of 25(OH)D may increase tissue levels of the

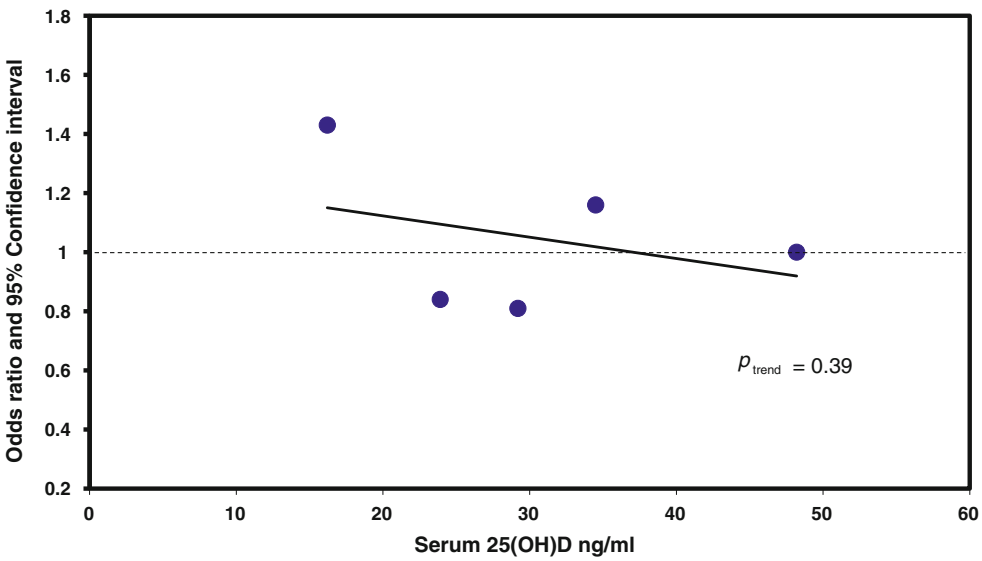

Fig. 1 Serum 25(OH)D level and risk of breast cancer, 664 white women, unmatched, and unadjusted for covariates

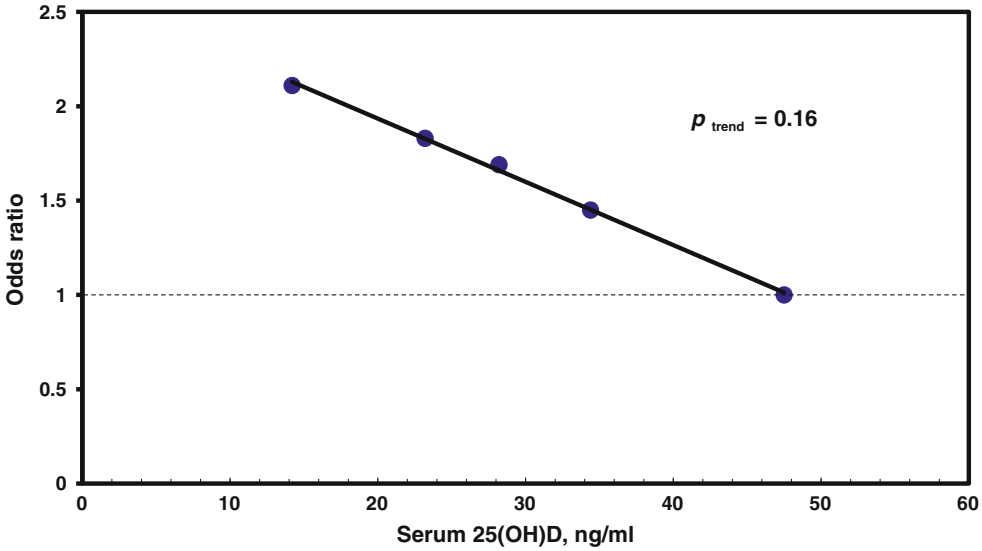

Fig. 2 Odds ratio for breast cancer, by serum 25(OH)D concentration, white women, blood drawn <90 days before diagnosis, unmatched, and unadjusted for covariates

most biologically active vitamin D metabolite, $1,25(OH)_2D$ [51]. While the majority of $1,25(OH)_2D$ synthesis occurs in the kidney and is homeostatically regulated, extrarenal synthesis of $1,25(OH)_2D$ can occur in a wide range of tissues, including the epithelial tissues of the breast [51]. Therefore, a high concentration of serum 25(OH)D may provide a greater amount of substrate for local synthesis of $1,25(OH)_2D$ in the breast epithelium [51]. In addition, vitamin D receptors that are highly sensitive to $1,25(OH)_2D$ [52] are found in normal breast epithelial cells.

$1,25(OH)_2D$ has the ability to promote differentiation and apoptosis in breast cancer cell lines [53, 54], providing a possible explanation for the apparent protective effect against breast cancer risk observed in observational studies. Moreover, the ability of $1,25(OH)_2D$ to induce differentiation and apoptosis, in addition to inhibiting angiogenesis, in existing breast cancer cells may explain the presence of an inverse association in several prior case–control studies where serum was collected for vitamin D measurement shortly after the diagnosis of breast cancer [19–26]. In an earlier case–control study performed by Janowsky and colleagues, white women in the lowest quartile of $1,25(OH)_2D$ had a risk of breast cancer that was 5.3 times (95 % CI 2.1, 13.4) higher than that of women in the highest quartile [55]. An inverse, albeit nonsignificant, association also was found in other studies [21, 31]. However, a study by Hiatt et al. [56] did not find an association between $1,25(OH)D_2$ and risk of breast cancer.

Strengths

The exposure of interest, serum 25(OH)D concentration, is a biomarker, making its ascertainment more precise than would have been possible through attempting to assess vitamin D status through a questionnaire. In addition, use of pre-diagnostic serum enabled the establishment of a temporal sequence.

The presence of selection bias, a major problem in case–control studies, was minimized in this study by not only matching controls to cases on important factors, but also because the study cohort was drawn from a military population, which, apart from having exposures common to all service members (such as diet and culture), has physical requirements and standards that apply equally to all members.

Limitations

This study had a number of major limitations. Cases were matched to controls on very few variables. Therefore, there is still the possibility of confounding by unmeasured breast cancer risk factors. This study utilized data from the DMSS, which contains data on hospitalizations and basic demographic information only. The data contained in the DMSS do not include information on subject's physical activity levels. As a result, we were unable to control for this variable in the regression analysis. Higher levels of physical activity have been associated with lower risk of breast cancer [57]. On the other hand, the protective effect of physical activity on breast cancer risk may be a consequence of higher concentrations of serum 25(OH)D due to increased sunlight exposure resulting from exercise performed outdoors. One case–control study of the relationship between ultraviolet sunlight exposure and breast cancer risk found a significant inverse association between sunlight exposure and breast cancer risk after adjustment

for physical activity and other confounders [58]. In another case–control study of 25-hydroxyvitamin D and breast cancer, serum 25(OH)D levels were still significantly, inversely associated with decreased risk of breast cancer after controlling for physical activity[23].

Previous research has suggested that the protective effect of serum 25(OH)D on breast cancer risk is greater in postmenopausal women [19–21, 33]. However, because our study population was relatively young (mean age 39.6 years), we were unable to assess any interaction between menopausal status and breast cancer risk. Furthermore, due to data limitations, we were unable to include several other variables of interest in studies of breast cancer risk such as parity, menarche, BMI, smoking, breast feeding, and family history of breast cancer. Therefore, we cannot rule out the possibility that results of the present study could have been influenced by some form of bias or a confounder that was not included in the analysis. However, this seems unlikely since serum was collected systematically in a military-wide surveillance program, where cultural factors tend to be similar as a result of military life.

Overall, there was not a statistically significant inverse relationship between serum 25(OH)D status and risk of breast cancer in this study. To date, results from case–control studies have been mixed. Most nested case–control studies in which serum was collected at a remote point in time far before diagnosis have not found an association between 25(OH)D and risk of breast cancer. On the other hand, findings from ordinary case–control studies in which serum is collected shortly before, during, or after diagnosis have been largely favorable to a benefit of 25(OH)D, which warrants further investigation. In the future, prospective cohort studies utilizing repeated 25(OH)D measurements, and especially randomized controlled trials of high dose vitamin D_3, should be undertaken to better assess the relationship that may exist between 25(OH)D and incidence of breast cancer.

Acknowledgments This research was supported by a Congressional allocation to the Penn State Cancer Institute of the Hershey Medical Center, Hershey, PA, through the Department of the Navy, Bureau of Medicine and Surgery, under Work Unit No. 60126 at the Naval Health Research Center (San Diego, CA, USA). The views presented here are solely the opinions of the authors and do not represent an official position of the Department of the Navy, the Department of Defense, or the U.S. Government. Approved for public release; distribution unlimited. Thanks to Dr. Angelee Ferber of the U.S. Army Medical Research and Development Command for providing the blood samples used in this study and for her outstanding support of the research. Thanks to June J. Kim, B.S., of Naval Health Research Center for her valued work editing the manuscript.

Appendix

See Figs. 3, 4 and 5

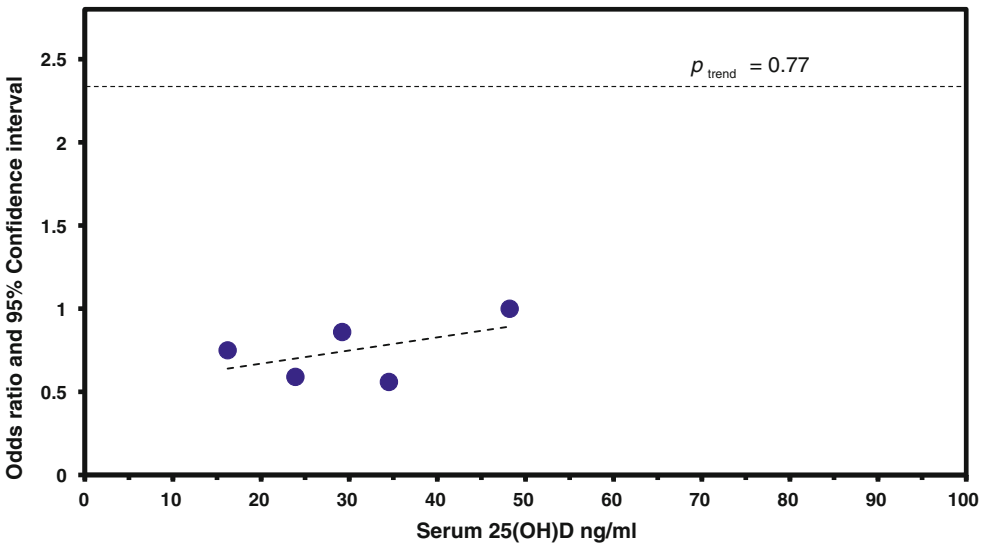

Fig. 3 Serum 25(OH)D level and risk of breast cancer, 536 nonwhite women, unmatched, and unadjusted for covariates

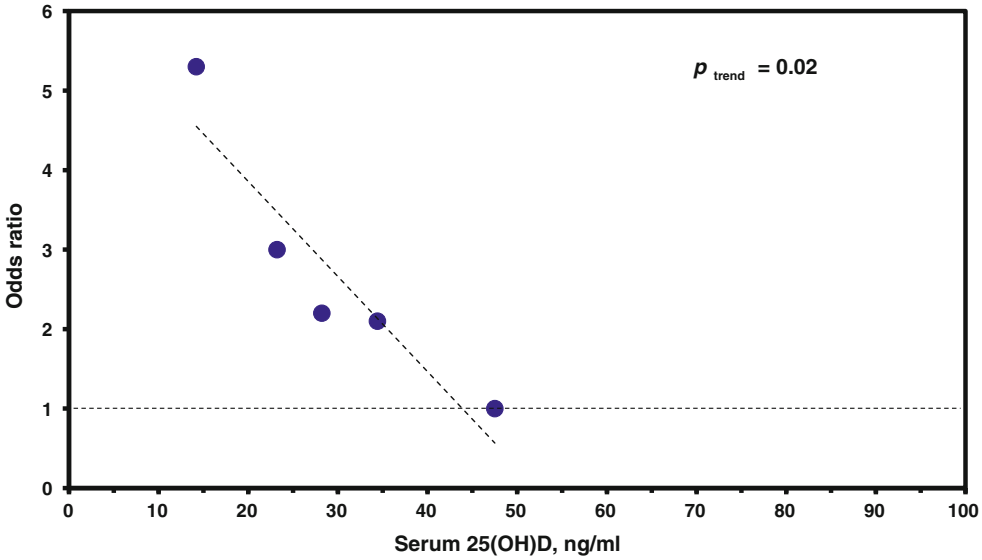

Fig. 4 Odds ratio for breast cancer, by serum 25(OH)D concentration, 108 nonwhite women, blood drawn <90 days before diagnosis, unmatched, and unadjusted for covariates

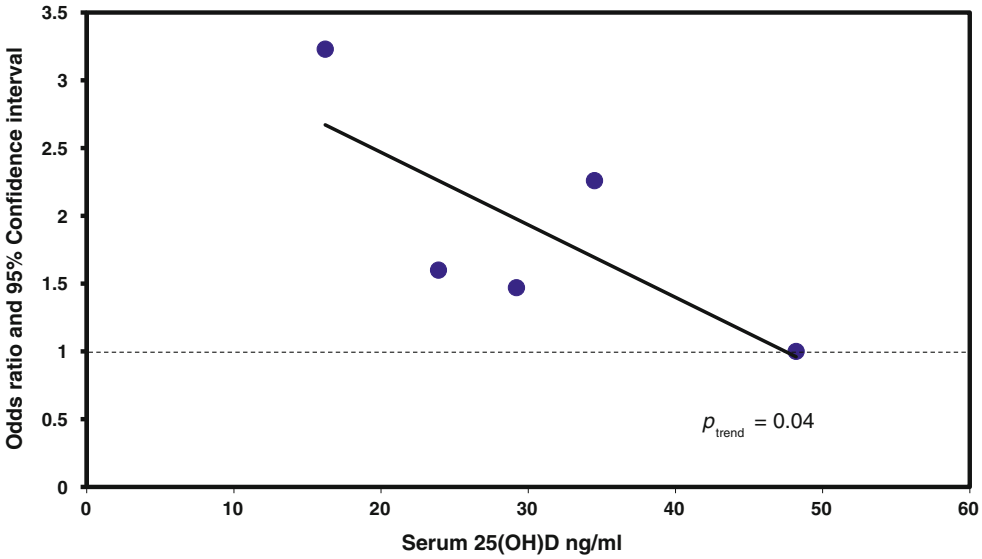

Fig. 5 Serum 25(OH)D level and risk of breast cancer, 248 women, blood drawn <90 days before diagnosis, unmatched, and unadjusted for covariates

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REPORT DOCUMENTATION PAGE

1. REPORT DATE (DD MM YY)
11/6/11

2. REPORT TYPE
Journal submission

3. DATES COVERED (from – to)
2010

4. TITLE
Serum 25-hydroxyvitamin D and Breast Cancer in the Military: A Case-control Study Utilizing Pre-diagnostic Serum

5a. Contract Number:
5b. Grant Number:
5c. Program Element Number:
5d. Project Number:
5e. Task Number:
5f. Work Unit Number: 60126

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8. PERFORMING ORGANIZATION REPORT NUMBER
12-21

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10. SPONSOR/MONITOR'S ACRONYM(S)
NMRC/BUMED

11. SPONSOR/MONITOR'S REPORT NUMBER(s)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES
Cancer Causes Control, 2013, 24, 495-504

14. ABSTRACT

Background. The objective of this study was to test whether a relationship exists between prediagnostic serum levels of 25-hydroxyvitamin D (25(OH)D) and subsequent risk of breast cancer in an active-duty U.S. military cohort.
Methods. 600 incident cases of female breast cancer were matched to 600 female controls as part of a nested case-control study. Conditional logistic regression was used to assess the relationship between serum 25(OH)D concentration and breast cancer risk, while controlling for race and age
Findings. The quintile cutpoints were < 15, 15-21.7, 27.4-35.1, and > 35.1 ng/ml.
There was a trend toward an inverse association between serum 25(OH)D and odds of breast cancer. In pairs in which serum was collected less than 90 days before diagnosis of the case, women in the highest quintile of serum 25(OH)D had 70% lower estimated risk of breast cancer (odds ratio 0.30, 95% confidence interval 0.12-0.74, $p \leq 0.01$ for contrast) compared to those in the lowest.
Interpretation. The favorable association of serum 25(OH)D with risk of breast cancer suggest that the influence of serum 25(OH)D in risk of breast cancer in adult women was strongest during the final few doublings of the tumor mass preceding diagnosis in this population. Further epidemiological research, including cohort studies, of the relationship between serum 25(OH)D status and breast cancer risk and its timing should be undertaken to confirm this association.

15. SUBJECT TERMS
25-hydroxyvitamin D, breast cancer, epidemiology, serum studies

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	18a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Commanding Officer
UNCL	UNCL	UNCL	UNCL	12	18b. TELEPHONE NUMBER (INCLUDING AREA CODE) COMM/DSN: (619) 553-8429